

## Interaction of Hepatitis C Virus-Like Particles and Cells: a Model System for Studying Viral Binding and Entry

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Hepatitis C virus-like particles (HCV-LPs) containing the structural proteins of HCV H77 strain (1a genotype) was used as a model for HCV virion to study virus-cell interaction. HCV-LPs showed a buoyant density of 1.17 to 1.22 g/cm<sup>3</sup> in a sucrose gradient and formed double-shelled particles 35 to 49 nm in diameter. Flow cytometry analysis by an indirect method (detection with anti-E2 antibody) and a direct method (use of dye-labeled HCV-LPs) showed that HCV-LPs binds to several human hepatic (primary hepatocytes, HepG2, HuH7, and NKNT-3) and T-cell (Molt-4) lines. HCV-LPs binding to cells occurred in a dose- and calcium-dependent manner and was not mediated by CD81. Scatchard plot analysis suggests the presence of two binding sites for HCV-LPs with high ( $K_d \sim 1 \mu\text{g/ml}$ ) and low ( $K_d \sim 50$  to  $60 \mu\text{g/ml}$ ) affinities of binding. Anti-E1 and -E2 antibodies inhibited HCV-LPs binding to cells. While preincubation of HCV-LPs with very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), or high-density lipoprotein (HDL) blocked its binding to cells, preincubation of cells with VLDL, LDL, HDL, or anti-LDL-R antibody did not. Confocal microscopy analysis showed that, after binding to cells, dye-labeled HCV-LPs were internalized into the cytoplasm. This process could be inhibited with anti-E1 or anti-E2 antibodies, suggesting that E1 and E2 proteins mediate HCV-LPs binding and, subsequently, their entry into cells. Altogether, our results indicate that HCV-LPs can be used to further characterize the mechanisms involved in the early steps of HCV infection.

Hepatitis C virus (HCV) is the major etiology of non-A, non-B hepatitis that infects 170 million people worldwide. Approximately 70 to 80% of HCV patients develop chronic hepatitis, 20 to 30% of which progress to liver cirrhosis (52). At present, there is no vaccine available to prevent HCV infection, and current therapies are not optimal. The initial steps of HCV infection (binding and entry) that are critical for tissue tropism, and hence pathogenesis, are poorly understood. Studies to elucidate this process have been hampered by the lack of robust cell culture systems or convenient small animal models that can support HCV infection.

HCV is an enveloped, positive-strand RNA virus that belongs to the *Flaviviridae* family. Based on the sequence heterogeneity of the genome, HCV is classified into six major genotypes and  $\sim 100$  subtypes (52). The viral genome ( $\sim 9.6$  kb) is translated into a single polyprotein of  $\sim 3,000$  amino acids (aa). A combination of host and viral proteases are involved in polyprotein processing to give at least nine different proteins (for a review, see reference 4). The structural proteins of HCV are believed to comprise the core protein ( $\sim 21$  kDa) and two envelope glycoproteins: E1 ( $\sim 31$  kDa) and E2 ( $\sim 70$  kDa). Like other enveloped viruses, E1 and E2 proteins most likely play a pivotal role in HCV life cycle: in the assembly of infectious particle and in the initiation of viral infection by binding to its cellular receptor(s). Since hepatocytes represent the pri-

mary site of HCV replication in vivo, the HCV genome has also been found in lymphoid cells. Infection of the lymphoid cells has been implicated in extrahepatic manifestations of HCV infection such as mixed cryoglobulinemia and B-lymphocyte proliferative disorders (2, 39, 42).

Detail ultrastructural features of the HCV virion remain elusive since direct visualization of virus particles from infected serum and tissues has proven to be difficult. Previous studies have shown that HCV particles vary in size between 30 and 60 nm in diameter (24, 38, 43). In addition, HCV particles display significant heterogeneity in buoyant density on sucrose density gradient centrifugation, ranging from low ( $<1.07$  g/ml) to high (1.25 g/ml) density (22, 24, 47, 54). The heterogeneity of the particle density has been attributed to the variability in size (44), nonenveloped nucleocapsid particles (28, 48), and an association with antibodies or  $\beta$ -lipoproteins (38, 47).

To date, the cellular receptor(s) for HCV remains controversial. The observations that HCV can infect both hepatic and lymphoid cells suggest that HCV may use different cellular receptors to access different cell types. However, the absence of an in vitro system that supports HCV replication and particle assembly has hampered studies to elucidate the early steps of HCV infection, i.e., virus binding and entry. Association of HCV virions with  $\beta$ -lipoproteins in plasma has raised the possibility that HCV may use low-density lipoprotein receptor (LDL-R) for viral entry (1, 33). Others have proposed that CD81, a cellular surface protein belonging to the tetraspanin protein superfamily, is the putative receptor for HCV based on the interaction of CD81 with recombinant truncated E2 protein of HCV 1a (37). Nevertheless, several studies have shown

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that by use of the truncated E2 protein alone may not accurately reflect interaction of HCV virion with cells. Both E1 and E2 glycoproteins are known to associate in two types of complexes: (i) heterodimers stabilized by noncovalent bonds, which presumably represents the prebudding form of the viral envelope, and (ii) high-molecular-mass disulfide-bonded aggregates representing the misfolded proteins (8, 11, 13). Indeed, using a pseudotype vesicular stomatitis virus (VSV) expressing either E1 or E2 protein, it has been shown that both proteins are required for efficient infection and fusion into target cells (29, 45). Furthermore, the HCV virion binds to mononuclear cell lines regardless of their CD81 expression, whereas recombinant E2 protein binds poorly because of the lack of CD81 (20).

In this study, we use hepatitis C virus-like particles (HCV-LPs) derived from H77 (1a genotype) (27) to determine whether HCV-LPs can be used as a model for studying the early events (binding and entry) of HCV infection. Our study demonstrates that HCV-LPs can gain entry into susceptible cells independent of CD81 and LDL receptor pathways.

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## MATERIALS AND METHODS

**Reagents and antibodies.** Bovine serum albumin (BSA), very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) were obtained from Sigma (St. Louis, Mo.). Coomassie Plus protein assay reagent was from Pierce, Rockford, Ill. Lipophilic dye [CellTracker CM-DiI, a chloromethylbenzamide derivative of DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanineiodine)] and SYTO 12 (SYTO) nucleic acid stain were from Molecular Probes (Eugene, Oreg.), and sterile glass chamber slides (Lab-Tek II) were from Nalge Nunc International (Rochester, N.Y.). Soluble glutathione *S*-transferase fusion protein of the large extracellular loop of human CD81 (LEL-CD81) (17) was a gift from S. Levy (Stanford, Calif.). Flow cytometric analysis was performed on a FACSCalibur apparatus (Becton Dickinson). The peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) and fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). Rabbit anti-human LDL-R IgG was from Maine Biotechnology Services (Portland, Maine), mouse anti-human LDL-R IgG was from Oncogene (Cambridge, Mass.), and mouse anti-human CD81 IgG1 was from PharMingen (San Diego, Calif.).

**Virus and cell lines.** The plasmid DNA containing the infectious HCV clone of H77 strain (p90/HCV FL-long pU) (27) was from M. Major and S. Feinstone (Food and Drug Administration, Bethesda, Md.). Hybridoma cells expressing anti-E1 monoclonal antibody (MAb) A4 (14) was from H. Greenberg (Stanford Medical School), and the anti-E2 MAbs AP33 and ALP98 have been described previously (34). Cryopreserved primary cultured human hepatocytes (PHH; Clonetics, BioWhittaker, Inc., Walkersville, Md.) were used on the same day after plating. Human hepatoma cell lines (HepG2 and HuH7) and human T-cell line (Molt-4) were obtained from the American Type Culture Collection (Rockville, Md.). NKNT-3, a reversible simian virus 40 T-antigen-immortalized human hepatocyte line (26), was from I. J. Fox (Omaha, Nebr.). Aro, a human follicular thyroid carcinoma cell line (15), was provided by G. J. Juillard (University of California at Los Angeles).

**Recombinant baculovirus constructs.** Plasmid containing the infectious clone of HCV of 1a genotype (H77 strain), p90/HCV FL-long pU (27), was used as a template to generate recombinant baculovirus coding for the structural HCV proteins: core, E1 and E2/p7 and an additional 63 nucleotides (nt) of the amino-terminal part of NS2. p90/HCV FL-long pU plasmid was digested with *Sma*I and *Tth*111 I, releasing a DNA fragment (nt 278 to 2831) corresponding to core, E1, and the E2/p7/N terminal of NS2, and subcloned between the *Sma*I and *Xba*I sites of pFastBac plasmid, allowing its expression under the control of a polyhedrin promoter (pFB90S). Plasmid pFB90S was used to generate recombinant bacu-

lovirus Bac.HCV.1a.S by using BAC-to-BAC Baculovirus Expression System (Gibco-BRL/Life Technologies, Gaithersburg, Md.) according to the manufacturer's protocols. The nucleotide sequences of the recombinant baculovirus were verified by restriction enzyme analysis and DNA sequencing. The virus titer was determined by BacPAK Baculovirus Rapid titer kit (Clontech, Palo Alto, Calif.). Expression of core, E1, and E2 proteins of the recombinant baculovirus in Sf9 cells (*Spodoptera frugiperda*) was analyzed by indirect immunofluorescence. As a negative control, pFastBac containing *gusA* gene was used to generate recombinant baculovirus expressing  $\beta$ -glucuronidase (Bac-GUS).

**Expression and purification of HCV-LPs.** The method for the expression and purification of HCV-LPs was modified from what was published previously for HCV-LP purification (5). Sf9 cells grown at 27°C in Sf900 medium (Gibco-BRL/Life Technologies) were infected with recombinant baculovirus at a multiplicity of infection of 5 to 10, and cells were harvested at day 3 postinfection. All purification steps were carried out on ice. Cells were washed once with ice-cold TNC buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM CaCl<sub>2</sub>) containing 1 mM Pefabloc SC and a cocktail of EDTA-free protease inhibitors (Roche, Indianapolis, Ind.) and resuspended in TNC buffer containing 0.25% digitonin and protease inhibitors. Cells were homogenized and allowed to sit on ice with gentle agitation and then monitored for cell lysis by trypan blue exclusion. The cell lysate was centrifuged to remove nuclear debris and plasma membrane, and the supernatant was pelleted over a 30% sucrose cushion. The pellet was resuspended in TNC buffer and applied onto 10.5 ml of a 20 to 60% sucrose gradient in SW41 tubes (Beckman) and centrifuged at 100,000  $\times$  g for 16 h. Fractions (1 ml) were collected from the top of the tube and tested for E1, E2, and core proteins by enzyme-linked immunosorbent assay (ELISA) (49) and Western blotting. Fractions containing HCV-LPs were stored at -70°C. Protein concentration was determined by using Coomassie Plus protein assay reagent (Pierce) with BSA as the protein standard. The ultrastructural morphology of HCV-LPs was analyzed by cryoelectron microscopy.

**Binding assay and Scatchard plot analysis.** The binding assay was performed at 4°C in 100  $\mu$ l of TNC buffer containing 1% BSA. Binding HCV-LPs to cells ( $2 \times 10^5$ ) was determined by two different methods: the indirect or direct labeling of HCV-LPs. For indirect labeling, cells were incubated with various amounts of HCV-LPs for 2 h and washed twice, and then the cells were incubated with anti-E2 MAb (AP33) (15  $\mu$ g/ml) for 30 min, followed by FITC-labeled goat anti-mouse IgG (4  $\mu$ g/ml) for 30 min. Cell-bound HCV-LPs was analyzed by flow cytometry. Nonspecific fluorescence was measured by adding primary and secondary antibodies in the absence of HCV-LP to cells. The mean fluorescence intensity (MFI) of bound HCV-LP was determined after subtraction of the nonspecific fluorescence value.

In the direct labeling, HCV-LPs were labeled with SYTO (nucleic acid dye) or CM-DiI (lipophilic dye) according to the manufacturer's protocol. HCV-LPs were incubated with a 5  $\mu$ M concentration of SYTO or a 1 to 5  $\mu$ M concentration of CM-DiI in TNC buffer at 4°C for 15 min and then repurified through a 30% sucrose cushion to remove the free dye. Cells were incubated with increasing concentrations of labeled HCV-LPs for 2 h at 4°C and washed twice, and the bound HCV-LPs were analyzed directly by flow cytometry. The MFI value of 100  $\mu$ g of HCV-LPs/ml in the absence of cells was used (41).

**Effect of anti-E1/E2 antibodies, CD81, VLDL, LDL, and HDL on HCV-LPs binding.** SYTO-labeled HCV-LPs were preincubated with increasing amounts of anti-E2 (AP33 and ALP98), anti-E1 (A4), or isotype (control) IgG for 2 h at 4°C. The HCV-LP-antibody mixtures were then incubated with cells for 1 h. After a washing step, cell-bound HCV-LPs were analyzed as described above. The effects of VLDL, LDL, and HDL on HCV-LPs binding were tested by preincubating cells with either VLDL, LDL, HDL, or anti-LDL-R IgG prior to the addition of HCV-LPs. Alternatively, HCV-LPs were preincubated with these lipoproteins before being added to the cells. Similarly, HCV-LPs or cells were preincubated with recombinant LEL-CD81 or with anti-CD81 MAb.

**Confocal microscopy.** HuH7 and NKNT-3 cells were incubated with CM-DiI or SYTO-labeled HCV-LPs at 4°C for 30 min, followed by incubation at 37°C for various time periods. The specificity of the internalization process was determined by preincubating dye-labeled HCV-LPs with anti-E1 and anti-E2 antibodies before they were added to the cells. As a negative control, cells were incubated with CM DiI- or SYTO-labeled preparation from cells infected with Bac-GUS. Alternatively, Aro cells were incubated with dye-labeled HCV-LPs. Cells were fixed with 4% paraformaldehyde, washed, and mounted with a DAPI (4',6'-diamidino-2-phenylindole)-antifade system. Cells were imaged on a Leica TCS SP laser-scanning confocal microscope mounted on a DMIRBE inverted epifluorescent microscope. SYTO and CM-DiI fluorescent dyes were excited by 499- nm and 553-nm laser lines, respectively, from a water-cooled argon laser (Coherent Laser). SYTO and CM-DiI fluorescence emissions were monitored at 519 and 570 nm, respectively.

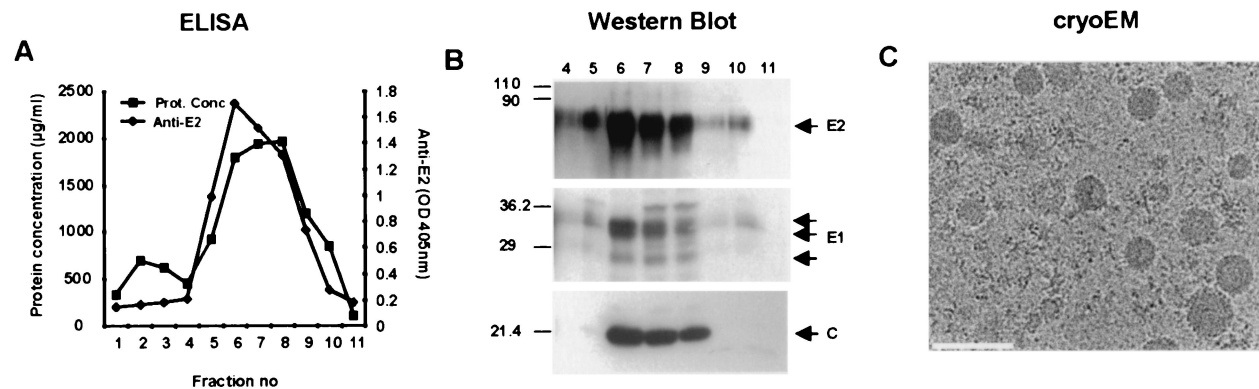


FIG. 1. Characterization of HCV-LP 1a. (A) HCV-LPs 1a were harvested on day 3 postinfection and purified as described in Materials and Methods. Eleven fractions (1 ml) were collected from the top and tested for E2 reactivity by ELISA. (B) Western blot analysis of HCV-LPs. The similar fractions collected from panel A were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot analysis with anti-E2 (ALP98), anti-E1 (A4), and anti-core (C1) MAb. (C) Cryoelectron micrograph of HCV-LP 1a. Bar, 200 nm.

RESULTS

**Characterization of HCV-LPs.** HCV-LPs 1a were harvested on day 3 postinfection by gentle permeabilization of cells with 0.25% digitonin. After fractionation of the HCV-LPs on an equilibrium sucrose gradient, each fraction was analyzed by ELISA and Western blotting for the presence of C, E1, and E2 proteins. The ELISA results showed that the peak of E2 reactivity was detected in fractions 6 to 8, which correspond to buoyant densities of 1.17 to 1.22 g/ml (Fig. 1A). Western blot

analysis revealed that these fractions contain an E2 protein band at ~70 kDa, three major bands of E1 (~33, 32, and ~28 kDa), and a core protein band at ~21 kDa (Fig. 1B). The presence of three bands of E1 protein reflects the different extent of N-linked glycosylation. As analyzed by cryoelectron microscopy, HCV-LPs are vary in sizes (35 to 49 nm in diameter) (Fig. 1C). This size difference, in part, may be due to the difference in the amount of E1 or E2 proteins incorporated into each type of particle (data not shown).

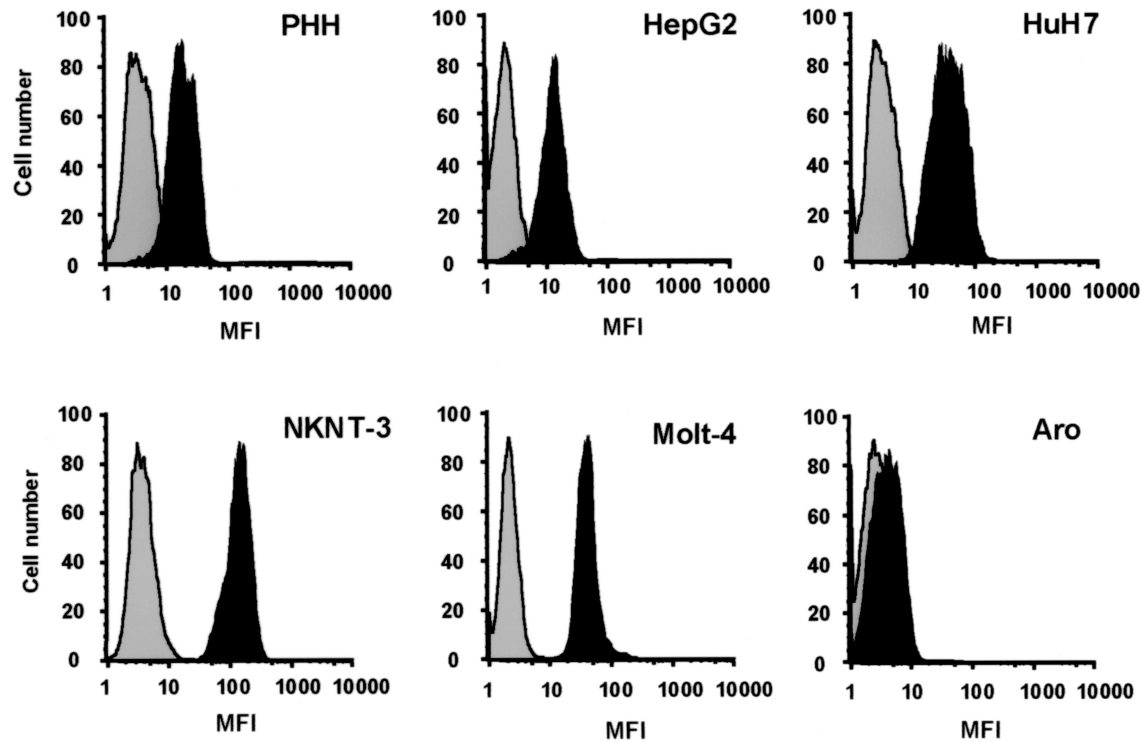


FIG. 2. HCV-LPs bind to human hepatic and T cells. Binding of HCV-LPs to human hepatic (primary human hepatocytes, HepG2, HuH7, and NKNT-3) and T (Molt-4) cells was detected by anti-E2 MAb, followed by FITC-labeled goat anti-mouse IgG (indirect method), as described in Materials and Methods; 2.5 µg of HCV-LPs was used for each binding study. Axes: x, MFI; y, number of cells. HCV-LPs did not bind to Aro, a human thyroid cell line.

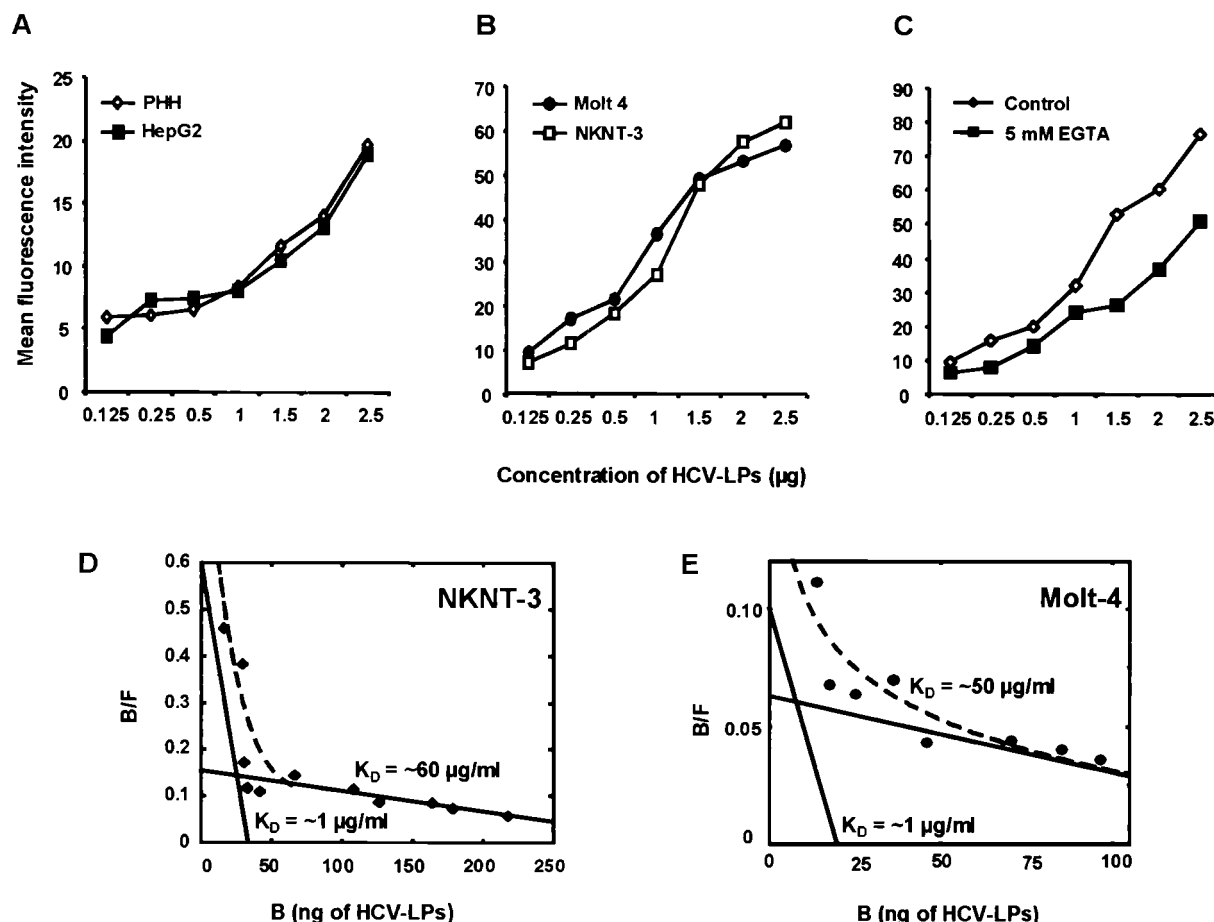


FIG. 3. Characteristics of HCV-LP binding to cells. (A and B) Dose-dependent binding. The binding of HCV-LPs to PHH, HepG2, NKNT-3, and Molt-4 cells was analyzed as in Fig. 2. Nonspecific fluorescence was measured by adding primary and secondary antibodies to cells in the absence of HCV-LPs. The MFI was determined after subtracting the nonspecific fluorescence value. The results presented are representative of three independent experiments. (C) Calcium-dependent binding. NKNT-3 cells and HCV-LPs were resuspended in 10 mM Tris-HCl-150 mM NaCl buffer containing 5 mM EGTA, and the binding assay was performed as described in the legend to Fig. 2. (D and E) Scatchard plot analysis of HCV-LP binding. SYTO-labeled HCV-LPs (1 to 200 μg/ml) were incubated with cells for 2 h at 4°C. After a washing step, cell-bound HCV-LPs were analyzed by flow cytometry. The ratios of bound (B) and free (F) HCV-LPs for each concentration were determined based on the MFI of 100 μg of HCV-LP/ml in the absence of cells regarded as total input (T).

**Binding of HCV-LPs to human hepatic and lymphoid cell lines.** Using HCV-LPs, we have developed a cell-based binding assay in two formats. The first is the indirect binding method, in which we use anti-E2 MAb to detect HCV-LP binding to cells. For the second format, HCV-LPs were labeled with a lipophilic (CM-DiI) or a nucleic acid (SYTO) dye and used for a direct binding assay. As a control for the direct binding assay, fraction prepared identically from control Bac-GUS-infected cells was labeled with the dye and used for the binding assay. The ability of HCV-LPs to bind various target cells was analyzed by flow cytometry first by the indirect method. As shown in Fig. 2, HCV-LPs bound to hepatic (PHH, HepG2, HuH7, and NKNT-3) and T-cell (Molt-4) lines, but not to thyroid cells (Aro). HCV-LPs also bound to human B-cell line (Daudi), but not to HeLa cells, mouse fibroblasts (3T3-L1), and mouse mastocytoma P815 cells (data not shown). Binding of HCV-LPs to target cells occurred in a dose-dependent manner and was saturable (Fig. 3A and B). HCV-LPs bound to Molt-4 and NKNT-3 cells with higher affinity than that to PHH and HepG2 cells.

Pretreatment of cells with 0.25% trypsin abolished HCV-LP binding (data not shown), suggesting that binding of HCV-LPs to cells is mediated by cellular surface protein(s). HCV-LPs binding to cells occurred, at least partially, in a calcium-dependent manner since the addition of 5 mM EGTA reduced this binding (Fig. 3C).

To estimate the affinity of HCV-LP binding to hepatic and lymphoid cells, Scatchard plot analysis was performed. Using the direct binding assay with SYTO-labeled HCV-LPs, we demonstrated the presence of a biphasic binding with high and low affinities to NKNT-3 and Molt-4 cells. The high-affinity binding site has a dissociation constants ( $K_d$ ) of ~1 μg/ml, while the lower-affinity binding site has a  $K_d$  of ~50 to 60 μg/ml (Fig. 3D and E).

**Inhibition of HCV-LP binding by anti-E1 and anti-E2 MAbs.** Binding of HCV-LPs to cells is most likely mediated through the envelope proteins E1 and E2. Preincubation of SYTO-labeled HCV-LPs with anti-E2 (AP33 or ALP98) or anti-E1 (A4) MAbs inhibited HCV-LP binding to cells in a dose-dependent manner. On the other hand, neither isotype

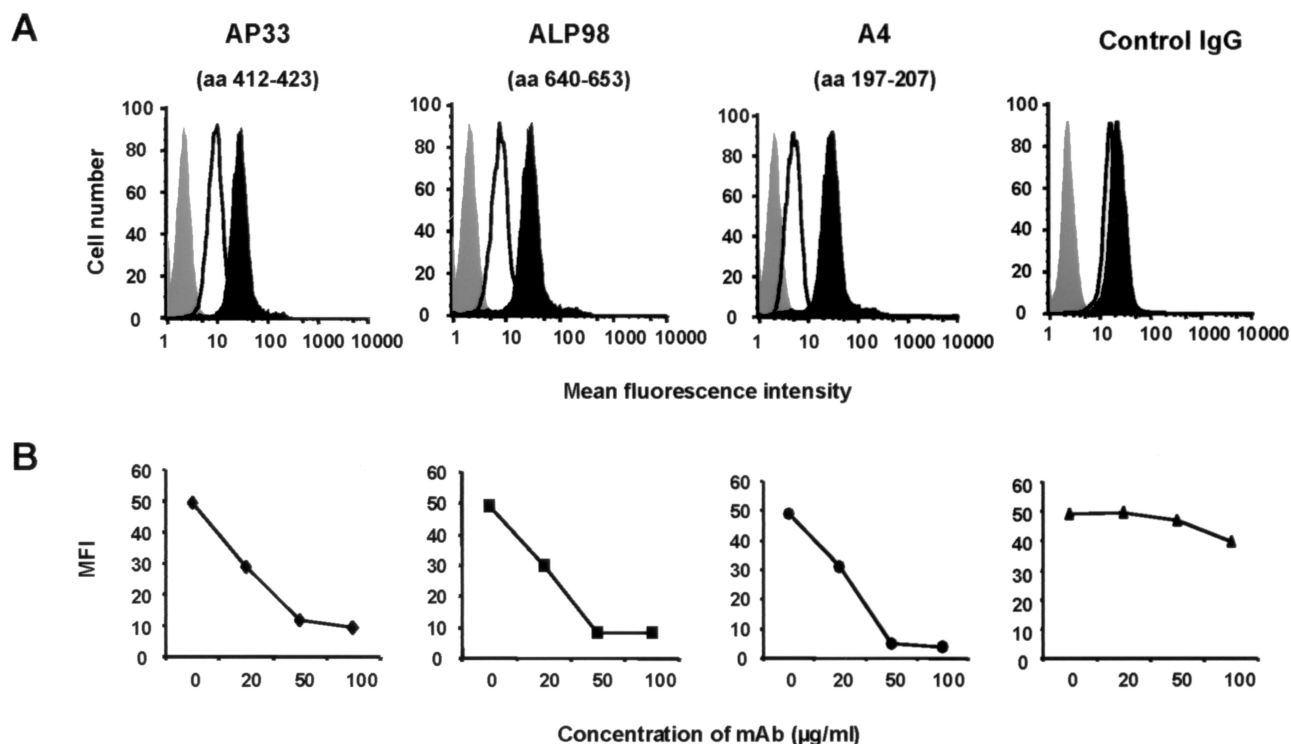


FIG. 4. Inhibition of HCV-LPs binding to cells by anti-E1 and anti-E2 antibodies. SYTO-labeled HCV-LPs were preincubated with 20 to 100  $\mu\text{g}$  of anti-E2 (AP33 and ALP98), anti-E1 (A4), or isotype control IgG/ml for 2 h at 4°C. The HCV-LP-antibody mixtures were then incubated with Molt-4 cells for 1 h. The cell-bound HCV-LPs were analyzed as described in the text. (A) Flow cytometry histogram of HCV-LPs binding in the presence (20  $\mu\text{g/ml}$ ) (open graph) or absence (black graph) of antibodies. The background binding is shown as the gray graph. (B) Dose-response inhibition of HCV-LP binding by the respective antibodies.

control IgG (Fig. 4) nor anti-core antibody (data not shown) had any effect.

**Effect of CD81 on HCV-LP binding.** Although HepG2, HuH7, NKNT-3, and Molt-4 cells all bound to HCV-LPs, significant differences in their CD81 expression existed. As assessed by reverse transcription-PCR, the strain of HepG2 cells used in the present study lacks CD81 expression, whereas others express CD81 (data not shown). Hence, HCV-LPs bound to HepG2 cells in a CD81-independent manner. Recombinant CD81 failed to inhibit HCV-LP binding to HuH7 cells, although it partially inhibited HCV-LPs binding to Molt-4 and NKNT-3 cells (Fig. 5A). Furthermore, anti-human CD81 MAb that had been shown to block truncated E2 binding to cells (17) did not have any significant effect on HCV-LP binding to HuH7 and Molt-4 cells (Fig. 5B).

**Effect of VLDL, LDL, and HDL on HCV-LP binding.** Molt-4 cells that express LDL-Rs and have been used previously to characterize HCV-cell interaction (54) were used here to evaluate the effect of VLDL, LDL, and HDL on HCV-LP binding. Using the indirect binding, we found that LDL inhibited HCV-LPs binding when added simultaneously to cells (Fig. 6A), whereas preincubation of HCV-LPs with LDL completely abolished their binding to cells (Fig. 6B). Previous study has proposed that association of HCV virions and  $\beta$ -lipoproteins in the plasma may mask the virions from circulating antibodies and, at the same time, represent one mechanism of HCV entry into cells, i.e., through the LDL receptor (33). There are two

explanations for this finding. LDL may bind to the HCV-LPs and inhibit their binding to cells; alternatively, LDL binding to HCV-LPs may hinder the accessibility of HCV-LPs to anti-E2 MAb used in this indirect binding method. To distinguish between these two possibilities, the direct binding method was used. Cells were incubated with SYTO-labeled HCV-LPs. As shown in Fig. 6C, preincubation of labeled HCV-LPs with LDL reduced their binding to Molt-4 cells by >50%. A similar phenomenon was observed when HCV-LPs was preincubated with VLDL or HDL. However, when cells were preincubated either with VLDL, LDL, or HDL before the addition of HCV-LPs, HCV-LP binding was only slightly increased (Fig. 6C). Altogether, these results indicate that preincubation of HCV-LPs with VLDL, LDL, and HDL resulted in a lipoprotein-HCV-LP complex that inhibited HCV-LP binding to cell. Second, the slight increase in HCV-LP binding after preincubation of cells with these lipoproteins suggested that HCV-LPs might also interact with cell-bound VLDL, LDL, or HDL, in addition to other cell surface molecule(s). This view was confirmed by the inability of two anti-LDL-R antibodies to significantly block HCV-LP binding (Fig. 6C).

**Internalization of labeled-HCV-LPs by hepatic cells.** We further examined whether binding of HCV-LPs to cells can be followed by entry. HuH7 and NKNT-3 cells were incubated with CM-DiI- or SYTO-labeled HCV-LPs, respectively, for 30 min at 4°C and then at 37°C for various time points. Figure 7 showed the internalization of CM-DiI-labeled HCV-LPs by

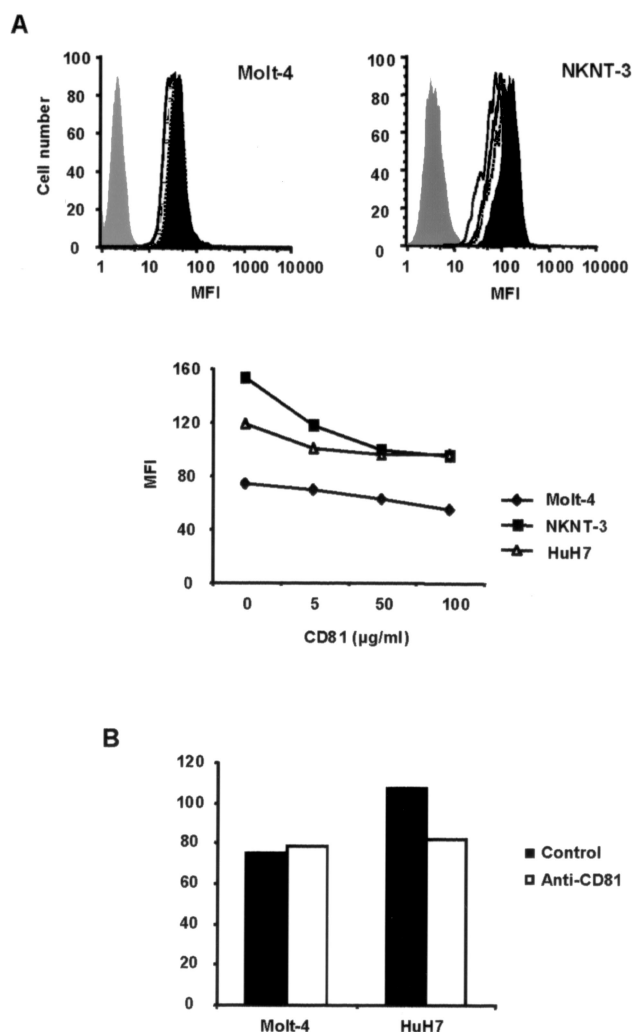


FIG. 5. Effect of CD81 on HCV-LP binding to cells. (A) Effect of human LEL-CD81 on HCV-LP binding. SYTO-labeled HCV-LPs were preincubated with increasing amounts of soluble human LEL-CD81 for 2 h at 4°C prior to addition to Molt-4, NKNT-3, or HuH7 cells. The binding assay was performed as in Fig. 3A. The top panel shows the flow histograms, and the bottom panel shows the MFIs. (B) Effect of anti-CD81 on HCV-LP binding. Molt-4 and HuH7 cells were preincubated with mouse anti-human CD81 IgG (20 μg/ml) for 2 h at 4°C, and then SYTO-labeled HCV-LPs were added, followed by further incubation for 1 h at 4°C. Cell-bound HCV-LPs were analyzed as described previously.

HuH7 cells as analyzed by laser-scanning confocal microscopy. This internalization is temperature dependent since only a weak signal was detected at 4°C (Fig. 7A) whereas, after incubation at 37°C, a higher intensity of dye-labeled HCV-LPs was observed in the cytoplasm surrounding the nucleus (Fig. 7B). In contrast, HuH7 cells did not uptake CM-DiI-labeled Bac-GUS preparation after incubation at 37°C (Fig. 7C). Aro cells that did not bind HCV-LPs (Fig. 2) were used as a negative control to assess the specificity of the internalization of HCV-LPs. As expected, Aro cells did not take up labeled HCV-LPs (data not shown).

The ability of NKNT-3 cells to internalize SYTO-labeled HCV-LPs is shown in Fig. 7D to H. After incubation at 4°C, a

weak signal of SYTO-labeled HCV-LPs was found mostly surrounding the cell surface (Fig. 7D). The incorporation of dye into the cytoplasm increased when cells were incubated at 37°C for 30 min (Fig. 7E). We also observed that SYTO dye was found in the nucleoli, which is presumably due to the staining of the RNA-containing nucleoli by the dye released from HCV-LPs after entry. NKNT-3 cells reacted poorly with SYTO-labeled Bac-GUS preparation (Fig. 7F). To assess whether specific antibodies could inhibit HCV-LP entry into cells, labeled HCV-LPs were preincubated with anti-E1 or anti-E2 antibodies for 2 h at 4°C. HCV-LPs (in the absence of antibodies) and after preincubation with antibodies were then incubated with cells for 15 min at 37°C. Although the control HCV-LPs were internalized by cells (Fig. 7G), preincubation with antibodies significantly reduced the incorporation of labeled HCV-LPs (Fig. 7H). These data suggest that E1 and E2 protein mediate HCV-LP binding and, subsequently, their entry into cells.

## DISCUSSION

One of the major impediments in studying the initial steps of HCV infection is the lack of a robust cell culture system for HCV propagation. In the absence of purified HCV particles, the baculovirus-derived HCV-LP (5) has provided a surrogate model for this purpose. Generation of virus-like particles by using a recombinant baculovirus expression system has been successfully reported for many viruses such as papillomavirus (30), rotavirus (23), and JC polyomavirus (19). These virus-like particles have been demonstrated to be a valuable tool for investigating virus-cell interaction of papillomavirus (10) and for identifying the candidate receptor for Norwalk virus (46).

In the present study, we generated HCV-LPs of 1a as a model for studying the mechanism of attachment and entry of HCV infection. The buoyant density of HCV-LPs (1.17 to 1.22 g/ml) resembles that of the high-density HCV particles in circulation (22, 24, 38, 54). It is believed that the low- and intermediate-density fractions represent HCV particles associated with  $\beta$ -lipoproteins (38, 47). Cryoelectron microscopy analysis featured HCV-LPs as double-shelled particles 35 to 50 nm in diameter. Of note, we also found that HCV-LP 1a is recognized by a panel of human and mouse anti-E1 and anti-E2 MAbs; some of these are conformation-sensitive anti-E2 MAbs, indicating that the E2 protein on HCV-LPs adopts a proper conformation (49).

HCV-LPs can bind to human hepatic and lymphoid cells that are believed to be the major sites of viral tropism *in vivo*. Binding of HCV-LPs to these cells was found to be dose dependent in saturated manner. Scatchard plot analysis of HCV-LP binding to hepatic and lymphoid cells indicates a biphasic binding with two different affinities, i.e., high- and low-affinity bindings. We estimated that the high-affinity binding site has a  $K_d$  of  $\sim 1$  μg/ml with at least 150 to 200 sites/cell, whereas the lower affinity has  $K_d$  of 50 to 60 μg/ml with  $>1,500$  sites/cell.

Depletion of calcium by 5 mM EGTA partially inhibited HCV-LP binding to cells, suggesting that one of the cellular receptors involved is a calcium-dependent type receptor. The requirement of calcium for virus binding has also been reported for pseudotype VSV bearing HCV E1 and E2 proteins (32) and rotavirus (40). The infectivity of pseudotype VSV-

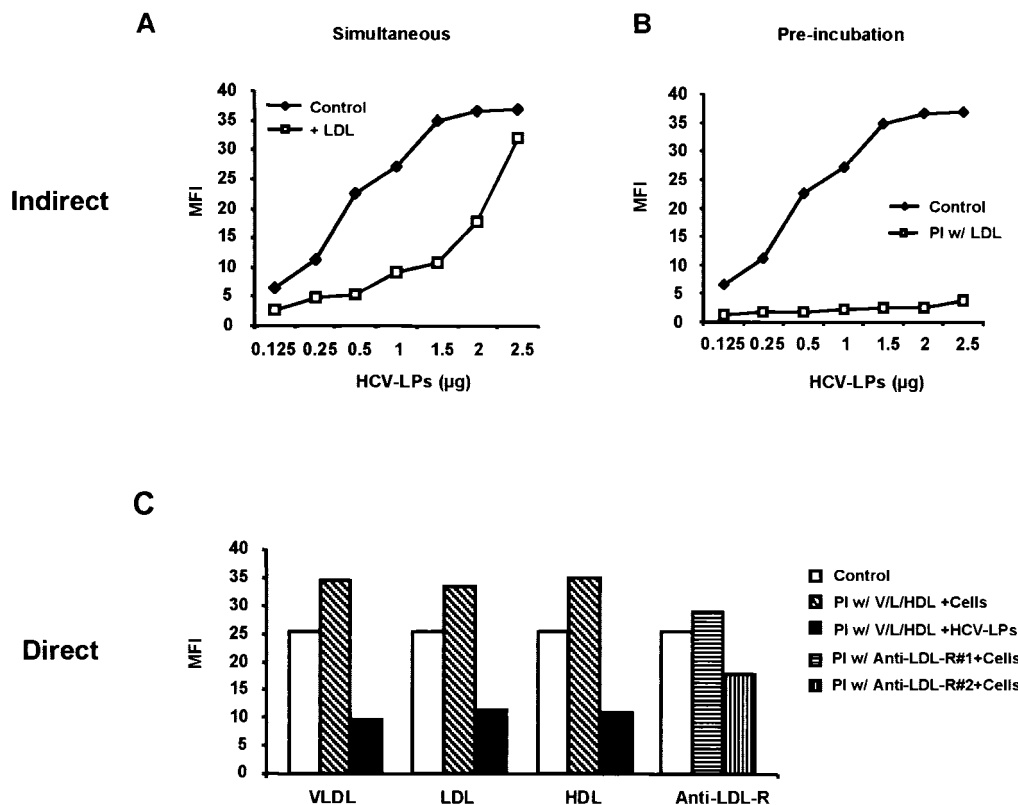


FIG. 6. Effect of VLDL, LDL, and HDL on HCV-LP binding to Molt-4 cells. Cell-bound HCV-LPs were analyzed by flow cytometry by the indirect method (A and B) or the direct method (C). (A) Increasing concentrations of HCV-LPs with or without LDL (0.5 mg/ml) were added simultaneously to the cells. (B) Alternatively, HCV-LPs were preincubated with LDL for 2 h at 4°C before being added to the cells. (C) SYTO-labeled HCV-LPs were incubated with cells for 1 h at 4°C, and cell-bound HCV-LPs were analyzed as described in Materials and Methods (open bars). Cells were preincubated with VLDL, LDL, or HDL (0.5 mg/ml) or anti-human LDL-R IgG (20 μg/ml) for 2 h at 4°C before the addition of SYTO-labeled HCV-LPs (hatched bars). Alternatively, SYTO-labeled HCV-LPs were preincubated with VLDL, LDL, or HDL at 4°C before being added to the cells (solid bar).

HCV E1 or E2 virus to HepG2 cells was reduced by up to ~50% in the presence of EGTA, suggesting that calcium is, in part, required for E1 or E2 binding to their receptor(s) (32). Many cellular receptors, including the LDL-R (3) and the ASGP-R (31), require calcium ions for their ligand-binding activity. Another possibility for the adverse effect of EGTA on HCV-LP binding maybe due to the importance of calcium ions for the conformation of E1 or E2 proteins. Calcium ion has been known to be important for particle assembly for many viruses, including herpesvirus (55), bovine papillomavirus (35), JC polyomavirus (19), rotavirus (12), and African swine fever virus (7).

Specific anti-E2 and anti-E1 antibodies can inhibit HCV-LP binding to cells in a dose-dependent manner, suggesting that both E1 and E2 proteins mediate binding of HCV-LPs to cells. Alternatively, the inhibitory effects exerted by these antibodies occurred through steric hindrance rather than via direct blocking of the receptor-binding site on the envelope proteins. Consistent with our findings, Garcia et al. (18) has recently identified six hepatocyte-binding sequences (HBSs) located on E1 and E2 by using a series of 20-mer, overlapping synthetic peptides corresponding to the entire length of E1 and E2 protein of HCV 1a genotype. Two of the HBSs in the E1 region were

mapped at the N-terminal (aa 192 to 211) and central (aa 242 to 261) regions, whereas the remaining four HBSs are clustered within E2: aa 384 to 404 (HVR-1), aa 444 to 483, aa 505 to 543, and aa 564 to 613. Interestingly, the anti-E1 (A4) and anti-E2 (AP33 and ALP98) MAbs that blocked HCV-LP binding recognize epitopes within or near these HBSs. A4 recognizes aa 197 to 207 (14), whereas AP33 and ALP98 recognize aa 412 to 423 and aa 640 to 653, respectively (34).

Virus-cell interaction is a multistep process and frequently involves more than a single receptor. There are, at least, three ways employed by virus to bind its target cells. First, virus can harbor two receptor-binding sites that allow binding to alternative receptors expressed on different cell types (e.g., adenovirus type 37) (53). Second, virus can bind to a "common" surface molecule that captures and concentrates virus at the cell surface, and this event is followed by binding to a high-affinity primary receptor (e.g., herpes simplex virus) (6). Third, virus binds to a high-affinity receptor, and this event induces conformational changes leading to the exposure of binding sites for a coreceptor (e.g., human immunodeficiency virus type 1) (16, 25). So far, little is known about which mechanism is adopted by HCV to bind and enter target cells. The association of CD81 and the LDL-R with E2 protein or HCV virion,

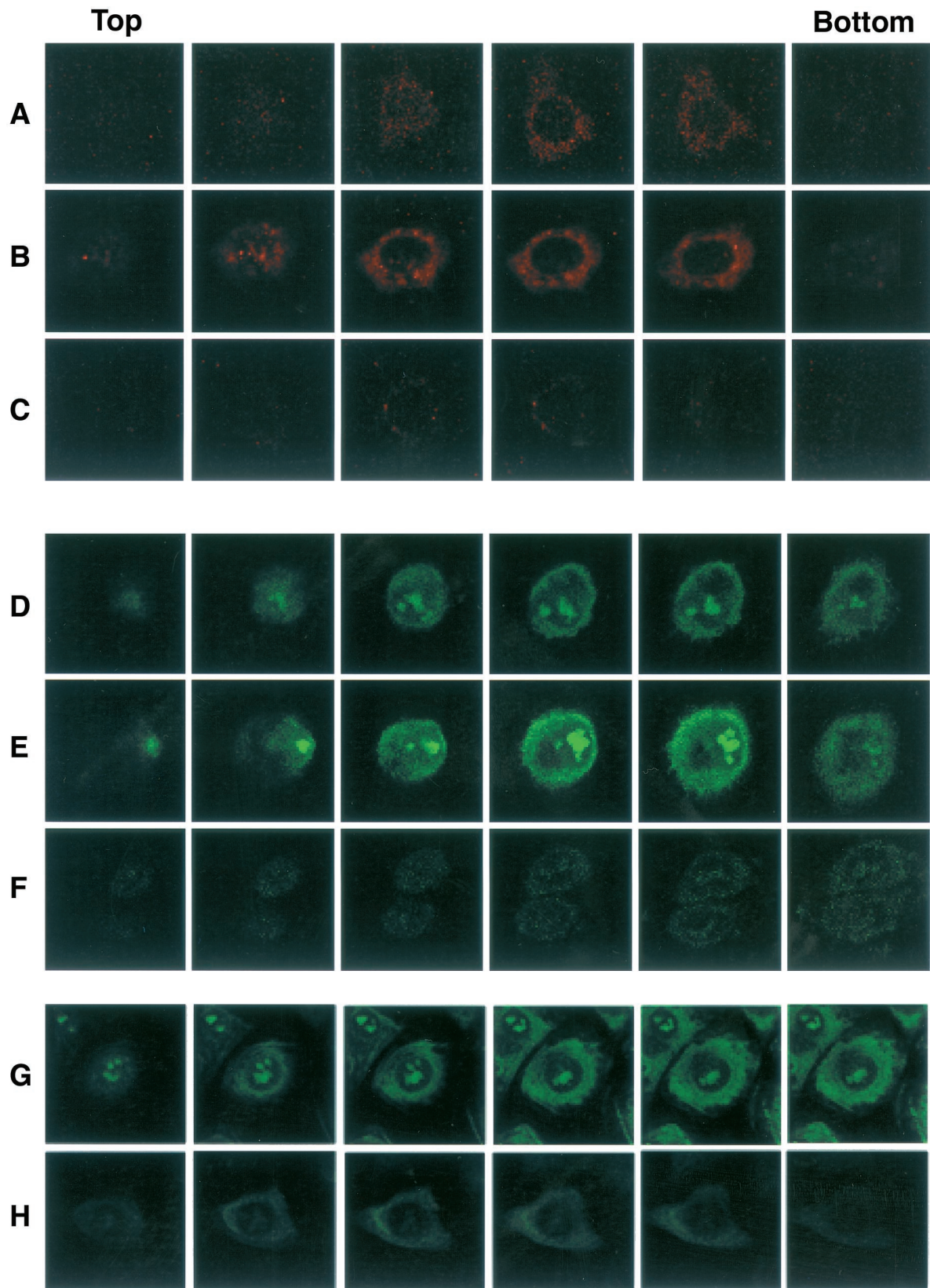


FIG. 7. Confocal microscopy analysis of labeled-HCV-LP internalization by cells. HuH7 cells were incubated with CM-DiI-labeled HCV-LPs at 4°C (A) and then at 37°C (B). As a negative control, cells were incubated with a CM-DiI-labeled control Bac-GUS preparation at 37°C (C). (D and E) NKNT-3 cells were incubated with SYTO-labeled HCV-LPs at 4°C (D) and then at 37°C for 30 min (E). (F) As a negative control, cells were incubated with SYTO-labeled Bac-GUS at 37°C for 30 min. (G) NKNT-3 cells were incubated with SYTO-labeled HCV-LPs for 15 min at 37°C. (H) Alternatively, cells were incubated with SYTO-labeled HCV-LPs that had been preincubated with anti-E1 or anti-E2 antibodies for 2 h. On each panel, six images representing the top to the bottom of the cells (left to right) are shown.

respectively, have led to the assumption that either one may represent the cellular receptor for HCV (1, 33, 37). Despite several reports demonstrating the E2 binding to CD81 (17, 21), the interaction between HCV virion with this molecule is less clear. CD81 molecule only inhibited the binding of truncated E2 protein, but not HCV virion, to Molt-4 cells (54), suggesting that the HCV virion may use other receptor(s) for entry into cells.

In the present study, we showed that HCV-LPs bind to various cell lines regardless of their CD81 expression. On CD81-positive cells (HuH7, NKNT-3, and Molt-4), blocking of cellular CD81 with anti-CD81 MAb did not inhibit HCV-LP binding. However, we found that recombinant CD81 partially reduced its binding to Molt-4 and NKNT-3 cells, but not to HuH7 cells. The different effects of CD81 on HCV-LP binding to different cell types is presumably due to the weak interaction observed between HCV-LPs and CD81 (49). It is possible that CD81-E2 interaction may serve some purpose other than viral attachment and/or entry. The poor ability of cellular CD81 to internalize bound ligands (36) also makes it unlikely as a sole determinant for viral attachment or entry. Recently, two independent studies demonstrated that engagement of CD81 with E2 protein led to the inhibition of natural killer cell activation, gamma interferon production and proliferation (9, 50). These results suggest that HCV may establish persistent infection through interaction of its E2 protein with the immune system.

The biological roles of lipoprotein-HCV interaction in the plasma of infected individuals remain unclear. In the present study we observed that the interaction between HCV-LPs and VLDL, LDL, or HDL might hinder HCV-LP recognition by the anti-E2 antibody. We also have preliminary data that LDL complexes with HCV-LPs by altering the sedimentation distribution of HCV-LPs in sucrose gradient centrifugation (not shown). These observations may have a biological relevance, i.e., lipoproteins could mask HCV virions from recognition by circulating antibodies. Our data corroborate the previous observation in which, after fractionation on a sucrose gradient, HCV-positive fractions with high infectivity in chimpanzee were found predominantly in low buoyant density (associated with lipoproteins) and could not be precipitated with an anti-HCV immunoglobulin antibody (22). In addition, we observed that preincubation of HCV-LPs with these lipoproteins inhibited HCV-LPs binding in a dose-dependent manner. Similar findings have also been reported for HCV virion (54) and pseudotype VSV expressing HCV E1 protein (32). The inhibition effect shown by these lipoproteins is more likely due to their interaction with HCV-LPs rather than competition for the cell surface receptor(s). This hypothesis is supported by the observation that preincubation of cells with VLDL, LDL, HDL, or anti-LDL-R did not inhibit HCV-LP binding. However, we cannot completely eliminate the possibility that the LDL/LDL-R pathway may play a minor role in mediating HCV-LP-cell interaction in this system.

As the mode of entry of HCV remains unknown, we sought to determine whether HCV-LPs could be used to elucidate this process. Incubation of cells with SYTO- or CM-DiI-labeled HCV-LPs at 37°C resulted in the incorporation of dye in the cytoplasm, suggesting the labeled-HCV-LPs were internalized. The internalization of labeled-HCV-LPs is shown to be specific for HuH7 and NKNT-3 cells and also HepG2 (data not

shown). Although we could not be absolutely sure that the incorporated dye inside the cell represents specifically the internalized HCV-LPs, several lines of evidence indicate that this is the case. First, preincubation of HCV-LPs with anti-E1 or anti-E2 antibodies significantly reduced the uptake of dye-labeled HCV-LPs by the cells, suggesting that E1 and E2 proteins mediate the particle binding and, subsequently, its entry into cell. Second, both HuH7 and NKNT-3 cells did not internalize dye-labeled Bac-GUS control preparation. Finally, dye-labeled HCV-LPs was not internalized by Aro cells that also did not bind HCV-LPs (not shown). In line with this study, Wellnitz et al. (51) has recently reported that HCV-LPs derived from H77c strain bind to hepatic and lymphoid cell lines. However, detailed characteristics of HCV-LP binding, i.e., the effects of calcium, anti-E1 antibody, lipoproteins, binding affinities, and the internalization process were not analyzed in that study.

Taken together, we have shown that HCV-LPs can be used as a valuable tool to study the mechanism of binding and entry of HCV infection. Further characterization of HCV-LP and its interaction with cells will help us understand the early steps of HCV infection and will facilitate studies to identify other candidate receptor(s) for HCV.

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